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Effects of Culture Methods on Survival, Weight, and Development of *Chironomus riparius* Meigen

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Chironomus riparius (Diptera:Chironomidae) is a tube-building midge which has four instars and is found in lentic-littoral waters. At the time of this study, there were no protocols for toxicity tests on specific chemicals in sediments and artificial substrates or for *C. riparius*. Before we could test for changes in sensitivity of larval *C. riparius* to Se-contaminated water and substrate (Beaty and Hendricks 2001), we needed a culture method that maximized survival, growth and development. Ristola (1995) reviewed the literature on feeding regimes for toxicity tests with chironomids, and found little consistency between studies. This makes it difficult to compare toxicity tests between studies because feeding regime affects toxicity. Anderson (1980) recommended that methods that meet the goals of each particular study rather than formalized methods be used. The objective of the present study was to determine how specific culture methods affected larval survival, growth and development. We tested effects of larval density, two water renewal methods, and tested for relationships between survival and the process of counting and between survival and egg hatch completeness.

MATERIALS AND METHODS

The *C. riparius* strain used in the present study was isolated from Stroubles Creek in Montgomery Co., Virginia by Jeff Kavanaugh at Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Larvae were cultured in 10 gallon aquaria with 5 L of water from Sinking Creek in Giles Co., Virginia. The aquaria were covered with screen tops to allow air space for mating. The pH of the creek water was 8.3 s. u., alkalinity was 117 ppm $CaCO_3$, hardness was 115 ppm $CaCO_3$ and conductivity was 197 μmhos. Creek water was vacuum filtered through 1.2 μm glass fiber filters to remove invertebrates and protozoa. The substrate was paper towels that had been autoclaved in water until there was no evidence of pigment leaching, and then washed in acetone until the acetone remained clear. The acetone was allowed to evaporate for 12 hr before the towels were used. Culture water was aerated, and larvae were fed a suspension of 10 g Tetra Growth Food (TetraWerke, Dr. rer. nat. Ulrich Baencsh GmbH, D 4520 Melle 1, Germany) (TGF), which had been ground in a blender with 100 ml distilled water to give a concentration of 100 g/L. While food was dispensed as a suspension, food quantities are reported as dry weight. Lab temperature remained between 21 and 24°C during all experiments, and

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the water was gently aerated. Eggs masses from the culture aquaria were hatched in watch glasses.

We examined the effects of larval density on survival and final larval weight by rearing 4 temporally successive groups of larvae for 14 d (larval density runs). Within 24 h of hatching, the larvae from an egg mass were pipetted into 250 ml Erlenmeyer flasks (surface area 32.17 cm^2) containing 100 ml water and one layer of cleaned paper towel. Run 1 initial densities consisted of 8 flasks of 5 larvae, 5 of 8, 4 of 11, 2 of 17, and 1 each of 25 and 31. Run 2 consisted of 7 flasks of 5 larvae, 1 of 6, 3 of 11, 1 of 12, and 1 of 38. Run 3 consisted of 1 flask of 50 and 2 of 150. Run 4 consisted of 2 flasks of 50 and 2 of 300. The number of larvae were changed in each run based on previous results to find levels that reduced survival. In runs one and two, each flask was fed 10 mg TGF at the start of the run. In runs three and four, each flask was fed 5 mg per larva at the start, then 1 mg per day to minimize the water fouling that occurred in experiments one and two. Water was not renewed. At the end of each experiment, larvae were dried for 24 h at 60 °C, stored in a desicator, counted and weighed on a Cahn 28 Automatic Electrobalance.

A problem encountered in the present study and others (e. g. Péry et al. 2002; Ristola et al. 1999) was how to measure larval density that changes over the course of an experiment due to mortality. Péry et al. (2002) used initial number of larvae per beaker as the measure of density in their analyses. To account for changing density, we fit a first-order least squares model, $Y=\alpha+\beta t$ where α is the Y intercept and β is the slope, to the initial and final number of larvae against time, then took the definite integral of the regression, $\int_0^{14} \alpha+\beta t\, dt = \alpha 14 + \frac{\beta 14^2}{2}$, to obtain the area under the curve. The unit for this measure is larval days.

Survival was regressed on larval days using a generalized linear mixed model (Wolfinger and O'Connell 1993) in which a binomial distribution was assumed and the logistic model $S = \frac{e^{\eta}}{1+e^{\eta}}$, where S is predicted survival and η is the linear predictor. A dispersion parameter φ was estimated. Values greater than 1 indicated over-dispersion relative to the binomial distribution and values less than 1 indicated under-dispersion. Flask assignment was modeled as a random effect, which was equivalent to treating each larva within a flask as an observation on that flask. This analysis was performed using the GLIMMIX macro for SAS version 8 (SAS Institute, Cary, NC, USA). Larval dry weight was regressed on larval days using a first-order least squares mixed model, again treating flask assignment as a random effect. This analysis was performed using SAS/STAT Version 8 PROC MIXED. The null hypothesis that the slope of the regression line was not significantly different from zero was tested by analysis of variance. Survival and dry weight were plotted with their regression functions and 95% confidence limits on the regressions, and are shown in Figure 1.

We compared survival and development in groups of larvae that were transferred to

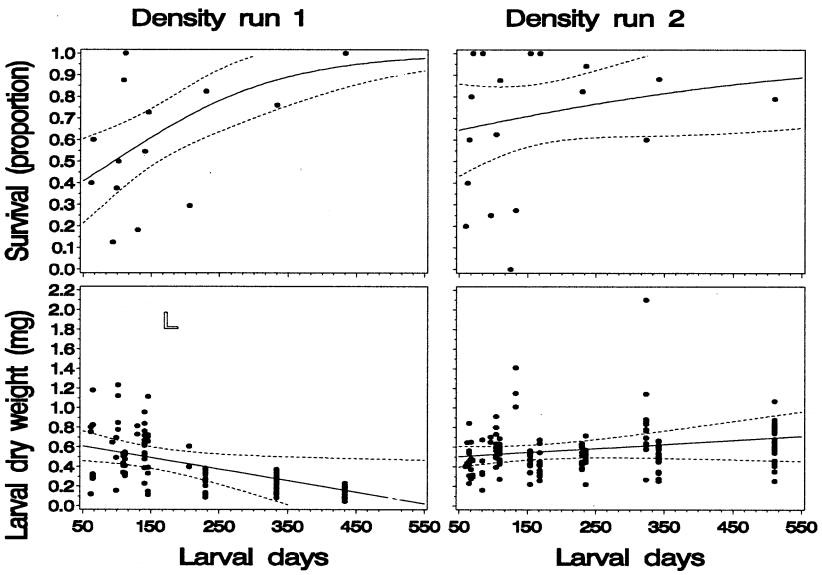

Figure 1. Dependence of larval survival and final dry mass on larval days. Larval days are the integral of a 1st-order regression of number of larvae per flask with respect to time. Solid lines are estimated survival or mass. Dashed lines are asymptotic 95% confidence limits on the regressions.

clean test vessels every 24 h (transfer runs) to that of larvae in which water was siphoned from the vessel and the vessel refilled every 24 h (siphon runs).

Development was measured as progression through instars over time. Instar number was determined by visual comparison of head capsule size. Larvae were pipetted within 24 h of hatching into 270 ml crystallizing dishes containing 200 ml water (63.62 cm^2 surface area). Runs 1 and 2, the transfer runs, consisted of 5 dishes containing 30 larvae. Runs 3 and 4, the siphon runs, consisted of 5 dishes containing 20 larvae and 3 of 30 larvae. At each transfer or siphoning, the dead were removed and the larvae were counted and fed 3 mg TGF on day 0 (start), 4 mg on day 1, 5 mg on day 2, 6 mg on day 3, 7 mg on day 4, 8 mg on day 5, 9 mg on day 6, 15 mg on days 7 through 10, and 20 mg on days 11 through 14.

Since the larvae built tubes out of food, and the presence of paper towels made recovery of young larvae difficult, no paper towel substrate was used. To check for a disturbance effect due to counting, two dishes in the third run were renewed daily without counting the larvae. The fourth run further tested for a relationship between survival and completeness of egg hatching in a particular egg mass. Three approximately equally-sized egg masses were selected and hatched separately, although the number of eggs in the masses was not counted.

Survival data are presented graphically in Figure 2 in terms of survivorship, which is the probability of any one individual surviving, or the per capita survival rate at any

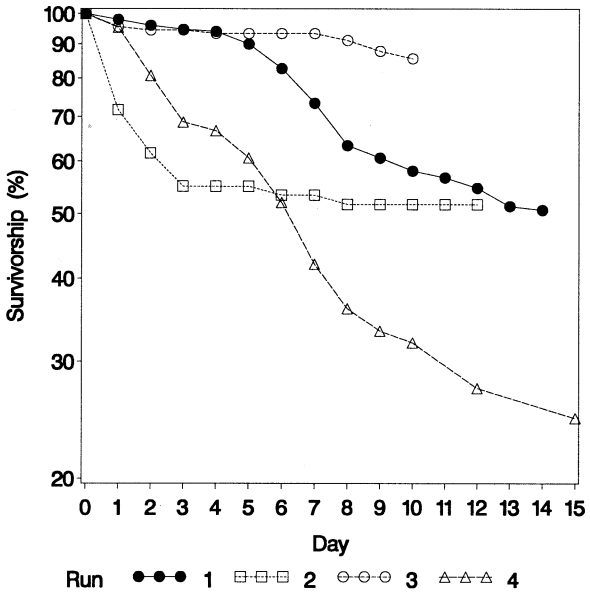

Figure 2. Pooled survivorship in each run of the water renewal method experiment. Larvae were transferred to a clean vessel daily in runs one and two. Water in vessels was replaced daily without moving the larvae in runs three and four.

age. To determine the most appropriate test for heterogeneity of survival curves (H_0:All curves are equal vs. H_A, All curves are not equal), the data were checked graphically for exponential and Weibull survival distributions. Empirical hazard functions were plotted and visually inspected to determine whether the ratios of hazard functions between groups appeared to be constant over time (Lee 1980). Because the survival distributions were neither exponential nor Weibull and were heterogeneous between trials, and hazard ratios were nonconstant, we used Gehan's generalized Wilcoxon test in PROC LIFETEST (SAS/STAT® Release 6.12 for Windows, SAS Institute Inc, Cary, North Carolina). Gehan's test is more powerful than other available tests under these conditions (Lee 1980). Heterogeneity of survival curves was tested between replicate dishes within trials, and between trials by pooling all data from replicate dishes.

RESULTS AND DISCUSSION

Female adult *C. riparius* attached cylindrical, gelatinous masses containing eggs to the aquaria sides at water level. Eggs were in a serpentine pattern, forming rows perpendicular to the gelatinous cylinder. Newly hatched larvae emerged from the egg masses by eating them. Afterwards, they built straight, randomly spaced tubes that were attached to the bottom of the vessels from food particles, and from paper fibers if supplied. Larvae consistently cleared TGF particles from an area around the tube.

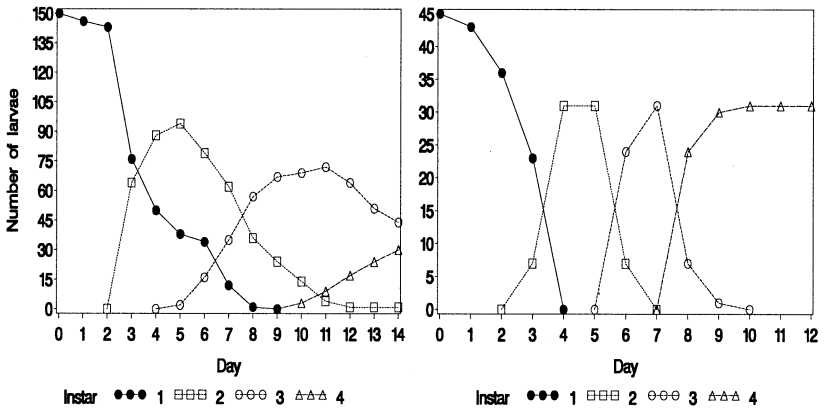

Figure 3. Number of larvae per instar per day in water renewal method experiment. Larvae were transferred to a clean vessel daily in run two. In run three, water in vessels was replaced daily without moving the larvae.

As they matured, they began to form clumps of tubes that housed up to five larvae, regardless of density. The tubes were usually longer than the larvae, and particles were never depleted. These tube clumps were often free from the vessel bottom, although some 3rd and 4th instar larvae continued to build tubes attached to the bottom of the glass. Tubes inhabited by 2 larvae were substantially longer than the 2 larvae combined and usually curved sharply in the space between the larvae. Larvae readily exited tubes when the tubes were gently prodded, whether or not the body was within the point of prodding. After leaving, larvae would attempt to enter the closest tube. If inhabited, they would search for another tube.

In density run one, increasing larval days caused a statistically significant increase in survival (η=-0.7793+0.008207**S*, *P*=0.0086, φ=0.8217) and a slightly significant decrease in final weight (Y=0.6677-0.00118**M*, *P*=0.0329). In density run two, increasing larval days did not affect survival (*P*=0.3146, φ=0.8446), or dry weight (*P*=0.1977). Data, regressions and 95% confidence limits on the regressions from runs one and two are plotted in Figure 1. In run three, larval days did not affect survival (*P*=0.2634, φ=1.0051) or dry weight (*P*=0.0910). In run four, larval days did not affect survival (*P*=0.1183, φ=0.9919), but was a highly significant factor (*P*<0.0001) affecting weight. At the minimum larval days, 666, estimated larval weight was 0.780 mg (95% c. l. = 0.7405 to 0.8190). At the maximum, 3833, estimated weight was 0.4296 mg (95% c. l. = 0.4029 to 0.4563). Water in the flasks initially containing 300 larvae was much more turbid than water in the lower density flasks, and had a strong sewage odor. The larvae weighed less and there was a higher percentage of third instars. Larval survivorship for pooled data from all dishes in the transfer runs are shown in Figure 2. At the 0.05 α level, survival functions were not equal among the four runs, but were equal between dishes within runs. The last point on each curve in Figure 2 indicates the day prior to the first pupation. Time to pupation decreased in each successive run. Survival in the non-counted vessels in

run 3, 8 out of 20 and 15 out of 20, was within the bounds of survival in the counted dishes, which ranged from 7 out of 20 to 15 out of 20, and the larval dry masses were not significantly different at the 0.05 α level. In run four, the 1st mass had five unhatched eggs, the 2nd had 99 unhatched eggs, and the 3rd had 58 unhatched eggs. There were no significant differences in survival between the larvae from the egg masses of different hatching success.

Transferring the larvae in the first two runs allowed us to search all material in the dishes for dead larvae. Some larvae were missing. In the 1st run, the percentage missing ranged from 33% to 70%. In the 2nd, the range was 56% to 89%. Leaving the tubes intact in the 3rd and 4th runs did not allow an extensive search for dead larvae. In the 1st and 2nd runs, 3 partially eaten larvae were recovered, and 2 dead larvae were found incorporated into tubes. The missing larvae were of all ages, and there was no one age where a higher proportion of the missing larvae were unaccounted-for. We did not observe cannibalism.

Larvae in run three, a siphon run, weighed more and developed faster than larvae in run two, a transfer run. The total number of larvae in all dishes in each instar per day for runs two and three is shown in Figure 3. The estimates of mean weight of the transfer group larvae was 0.209 mg (s.e. = 0.135), which was significantly less ($P<0.0001$) than the mean weight of the siphon group larvae, 1.164 mg (s.e. = 0.045). Siphon run larvae developed more rapidly and molts were more synchronous than in the transfer runs. In the siphon run, the numbers of larvae per instar by day were symmetrically distributed, where second instars in the transfer group were distributed with a right-skew.

A density-dependent increase in survival was observed only in run one. This was unexpected and not supported by the other runs. We were unable to find any other reports of density-dependent increases in survival of aquatic diptera larvae. We correctly estimated the correlations in the survival models because the dispersion parameters were nearly 1. We found no evidence that larval dry weight was density dependent in runs two and three. Ristola et al. (1999) found similarly ambiguous results. Our starting densities ranged from 0.155 to 9.325 larvae/cm^2, while their highest starting density was 0.417. They found a density-dependent increase in final weight, which they attributed to their holding food per larva constant throughout test vessels, so that food per area decreased as density decreased. Comparing our methods revealed a difficulty in designing density-dependence experiments. In runs one and two, we put the same weight of food in each flask, while runs three and four were fed on a per larva basis. Feeding per larva creates a lower food density, while feeding per area risks changing the environment through decomposition of uneaten food. This has serious implications for toxicity testing, since food level can change the bioavailability of substances in the water and sediment. The turbidity and sewage odor of the water in run three indicated that food levels were too high. It is possible that the differences in amount of food contributed to differences in relationships of weight and survival to larval days, especially since more total food was given in run three.

We chose dry weight as a response variable rather than growth as recommended by USEPA (2000). Since growth is the difference between mean final and initial weight, it requires estimation of an additional parameter, which adds variance to the model. Use of larval days was somewhat trivial in the present study since we only had initial and final number of larvae. Had we measured survival several times during the runs, we could have used numerical integration techniques such as the trapezoidal or Simpson's rule. In the present study, we could have just as well averaged the initial and final number, but chose to use larval days to facilitate comparisons to other studies.

We attempted to create a balanced design by using approximately equal numbers of larvae in each fixed treatment. To head off criticism that the experiments were pseudoreplicated, we must point out a flaw in Hurlbert's monograph (1984). He stated "...if "replicates" are only samples from a single experimental unit ..., then replicates are not independent..." and he follows by stating that such an experiment is pseudoreplicated. The basis for his argument is the fallacy that only observations whose errors are not independent are truly replicates, and that these can be the only sources of variance in a model. The purpose of a mixed model is to separate fixed effects, larval days in the present study, from repeated or random effects, which are flasks in the present study. Modeling repeated effects accounts for covariances that are created by having repeated observations on experimental units. An equivalent approach is to consider that one does not distinguish between flasks, which makes flask assignment a random effect. Hurlbert's (1984) concern that modeling random effects or repeated measures as if they were fixed effects will underestimate variance and lead to higher-than-nominal α levels is correct. Modeling the random effect with one covariance among all observations increased the estimated variance to the point that only the most dramatic differences in larval days detected weight as a significant fixed effect.

There were significant differences in survival functions between runs in the water renewal experiment, and the larvae gave tube-building energetic priority over development. Test conditions were identical between runs one and two, and between runs three and four. The decrease in development time from run three to run four may be attributable to a slight increase in temperature in the laboratory. Temperature in the laboratory fluctuated between 21 and 24°C throughout the year, but daytime temperatures were between 23 and 24°C during run four, and were between 21 and 23°C during run three.

Larvae in run two, a transfer run, developed slower than larvae in run three. We inferred that availability of tubes and building materials strongly affected growth and development rate. Péry et al. (2002) showed that under severe food limitation ($\leq$0.15 mg/larva/d), *C. riparius* larval development and emergence was delayed. It appeared that the larvae were not negatively affected by counting since there was no increase in survival or time to pupation in the uncounted dishes in run 4. We found that *C. riparius* met U. S. EPA's (2000) requirement of 70% survival when building tubes from food particles, so deep substrate may not be needed in chironomid toxicity tests. The ability of *C. riparius* in the present study to build tubes and pupal cases from

TGF may be an advantage in a toxicity test treatment where the experimenter wants to minimize or eliminate substrate without stressing the larvae, or to limit the types of material in the test vessel. We conclude that culturing thirty organisms at 20° to 25° in 270 ml crystallizing dishes containing 200 ml water, siphoned and renewed daily, maximized survival, growth and rate of development. Up to 20mg TGF, the food, can be added daily. The exact amount should provide excess material for tube-building but not foul the water. To facilitate toxicity testing, the tubes can prodded to make the larvae exit for counting as long as the tubes are left intact.

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